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## **Introduction**

The principal objective of this research is the development of the combination of hyperthermia (HT) and high dose rate (HDR) brachytherapy as a therapy for locally advanced, recurrent prostate cancer after failure using front line external beam definitive radiation therapy (EBRT). We had previously developed a system for low dose rate systems (LDR), however technological advances in HDR systems make the application of LDR essentially obsolete. There are several fundamentally different aspects to HDR practice and dosimetry, which render the LDR technology developed here obsolete as well. As a result the first three tasks in the statement of work are the development of a new template system, new software to control power deposition in the tumor and phantom testing before beginning patient treatment. As is demonstrated in the body of this report all three tasks have been completed on schedule. The new HDR template design differs radically from the older LDR system is easier to set up and more comfortable for the patient. The software was completely re-written to accommodate the fundamentally different HDR dosimetric approach. The combination of the hardware and software was then extensively tested on phantoms. Once satisfied that the system was safe for human application one patient was treated in accordance with the approved protocol during year 1. The final task is the development of heat activated gene therapy as an adjuvant to HDR brachytherapy specifically applied to this proposal but which should have considerably broader applicability.

## **Body of Report**

**Task 1** Complete the ongoing constructions of an electronic template interface compatible with HDR brachytherapy and hyperthermia systems, permitting simultaneous operation.

Completed during year 1 (see 2002 annual report)

**Task 2.** Complete the ongoing development of the computer code required to effectively drive the "random placement" needle patterns associated with current HDR brachytherapy practice.

### **Modifications to the Temperature Server (TS) and Master Treatment Control Server (MTCS)**

#### **Problem**

The original hardware/software specification called for the Temperature Server Application (TS) to have the ability to reside on a standalone computer system. Several issues arose concerning this configuration.

1. Increase setup complexity
2. Increased software complexity
3. Increased complexity for the operator
4. A very remote possibility of using one patient's temperature information to control the power deposition for another patient.

#### **Solution**

The MTCS application and the TS application are required to be executing on the same computer. Furthermore, they will continue to communicate using TCP/IP and our custom network protocol using an identical IP address.

MTCS is solely responsible for connecting to and controlling the TS. MTCS also becomes the only application that can disseminate temperature information. Accordingly the client application is required to establish a connection with only one application, the MTCS.

The TS will only accept one connection this reduces the complexity of the TS software resulting in a more robust application.

The TS handles verification that the correct MTCS is connecting to it by comparing the connecting applications IP address with it's own. If the IP addresses do not match the connection is refused.

### Assorted other Modifications

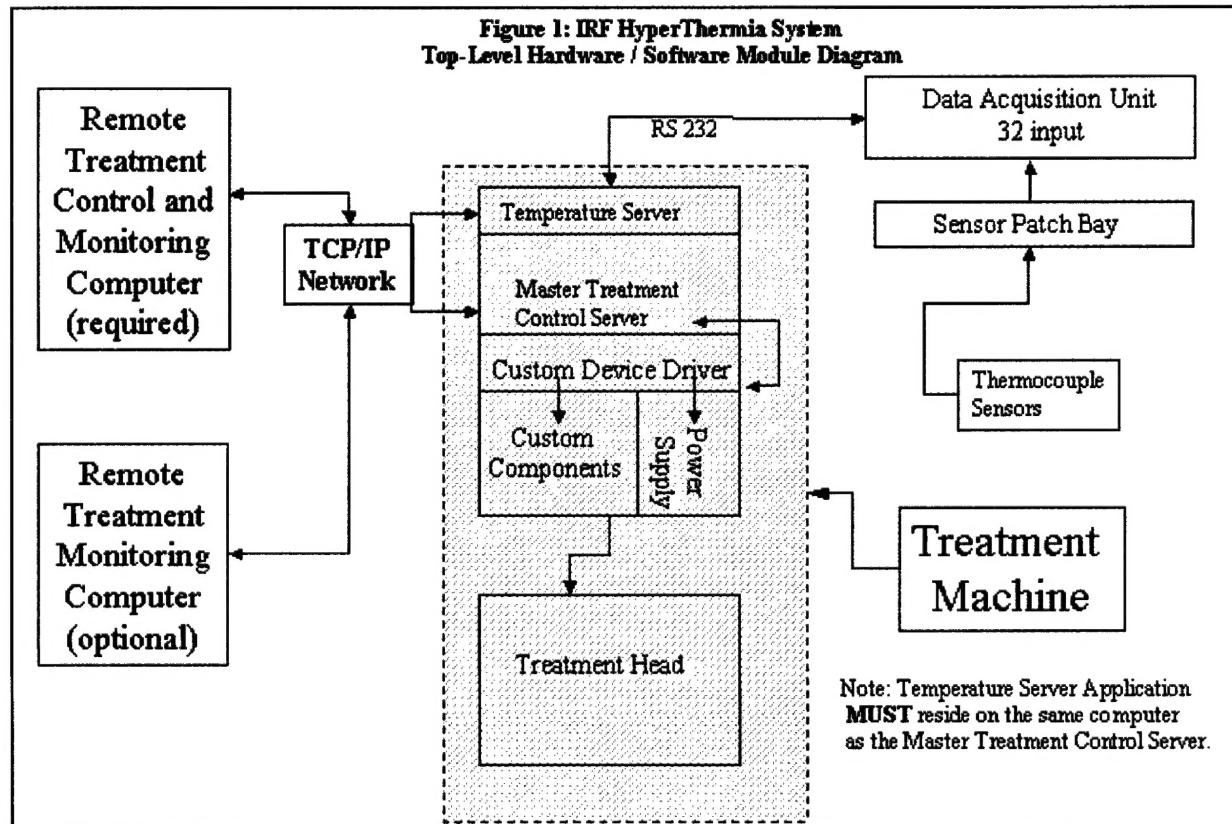
The client application controlling the treatment is referred to as the ICC. In prior versions it used operated as the Master Controller and included the responsibility to accept connections from observer client and disseminate information. That responsibility now resides with MTCS. The ICC accepts no connections from remote computers.

Benefits:      Reduced software complexity  
                    Increased robustness  
                    Quicker response time to user input

The ICC is no longer responsible for reading the setup file and then 'uploading' it to MTCS. Each application is responsible for reading the setup file on its own. In addition when ICC connects to the MTCS a check is made to be sure they are using the same setup file.

The setup file is now binary. Complete objects are written to and read from this file. Again this reduced software complexity and increases the software robustness and response time.

Enhanced data logging capabilities were implemented. All treatment related data can be written to any storage device on the network.



**Task 3.** Test the system on phantoms that are constructed to mimic patients that have undergone HDR prostate treatment and estimate how effectively it will generate the desired hyperthermia heating patterns.

Extensive testing of the modified software, similar to that reported in the year 1 annual report, was carried out and all operation was found to be within nominal parameters.

**Task 4.** Recruit and treat patients with recurrent prostate cancer that meet the patient selection criteria outlined in the approved clinical protocol.

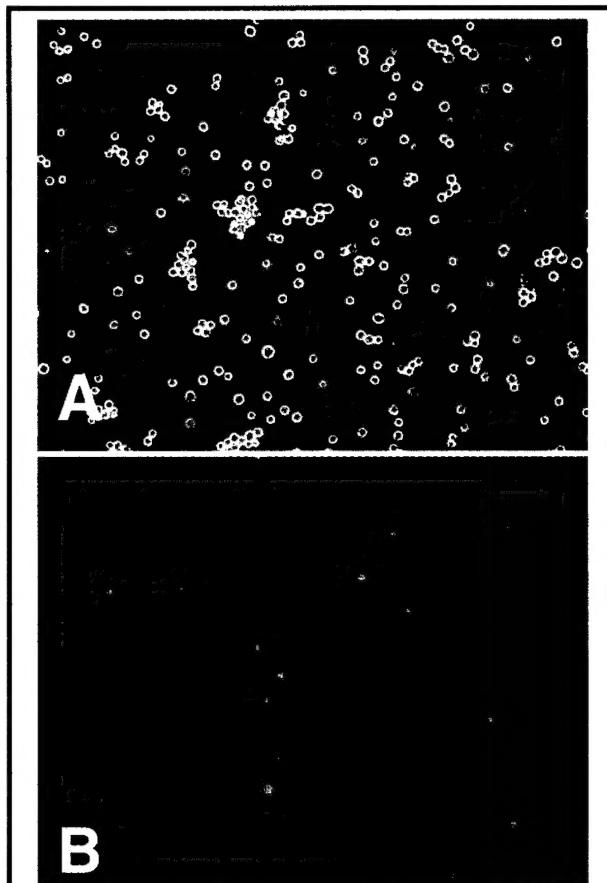
Four patients were evaluated and found to be appropriate candidates for this investigational therapy. Three of the patients declined after reviewing all available material and the informed consent. One patient signed the informed consent, however, on the treatment date admission laboratory tests showed abnormalities in kidney function which, in the opinion of the attending physicians, constituted an unacceptable risk to carrying out the procedure. As a result no patients were treated during year 2.

The patient treated during year 1 has responded well and shown no evidence of any chronic toxicity. This patient has now been followed for approximately 18 months and the PSA values from this follow-up period are as follows:

Date	PSA Value
11/26/2001 (treatment)	5.4
12/11/2001 (follow-up)	0.0
01/09/2002 (follow-up)	0.0
08/02/2002 (follow-up)	0.0
11/04/2002 (follow-up)	0.0
05/29/2003 (follow-up)	0.4

This result represent an excellent response but is inconclusive in itself. We have requested a change in the protocol to include not only patients that have failed on front line external beam therapy, but also those that have failed on front line seed implant therapy. This protocol change has been approved by our institutional IRB and is awaiting approval from the Army IRB before patients can be accrued (see attachment). It is hoped that this move will expand the potential patient population and improve accrual during year 3. Had this change been in place previously, two additional patients could have been accrued during the past three months.

**Task 5.** Investigation and integration of gene therapy into the treatment scheme.



**Fig. 2: Apoptosis Assay Following CdtB Treatment in an Anaerobic Chamber**

Dut-145 prostate cancer cells were treated with a fluorescent indicator of caspase activity while they expressed CdtB in an anaerobic chamber. The assay was performed 24 h following heat shock induction (41.0°C 1 h) of CdtB in cells that were infected (MOI 5) with the CdtB adenovirus. The phase contrast (A) and fluorescence (B) micrographs are of the same field. The green fluorescence in B indicated caspase activity and, putatively, apoptosis in those cells.

After performing the apoptosis assay, cells were trypsinized in the anaerobic chamber and suspended in PBS. Some of the cell suspension was used for microscopic observation while the rest was analyzed with flow cytometry.

One of the primary goals of the overall research project is the development of heat activated gene therapy vectors that could be activated by and integrated into the combined therapy to potentially augment local control of the prostate cancers under treatment. Bacterial proteotoxins were proposed as the toxic agent to be incorporated into these vectors. In the report for the previous years funding (year 1) we reported the development of a combined heat-radiation double promoter system. The primary purpose of this double trigger molecular system was two fold. In the first instance it would permit us to more easily make adenoviral vectors, which had proved extremely difficult for the initially proposed use of the Shigella and Diphtheria toxins. In the second instance the double switch promoter would provide additional control of the proteotoxin expression in the event that the vector should escape the tumor and travel to other parts of the body via the blood stream. This achievement was an important step in formulating a series of molecular tools that can be used in the development of potentially applicable clinical agents.

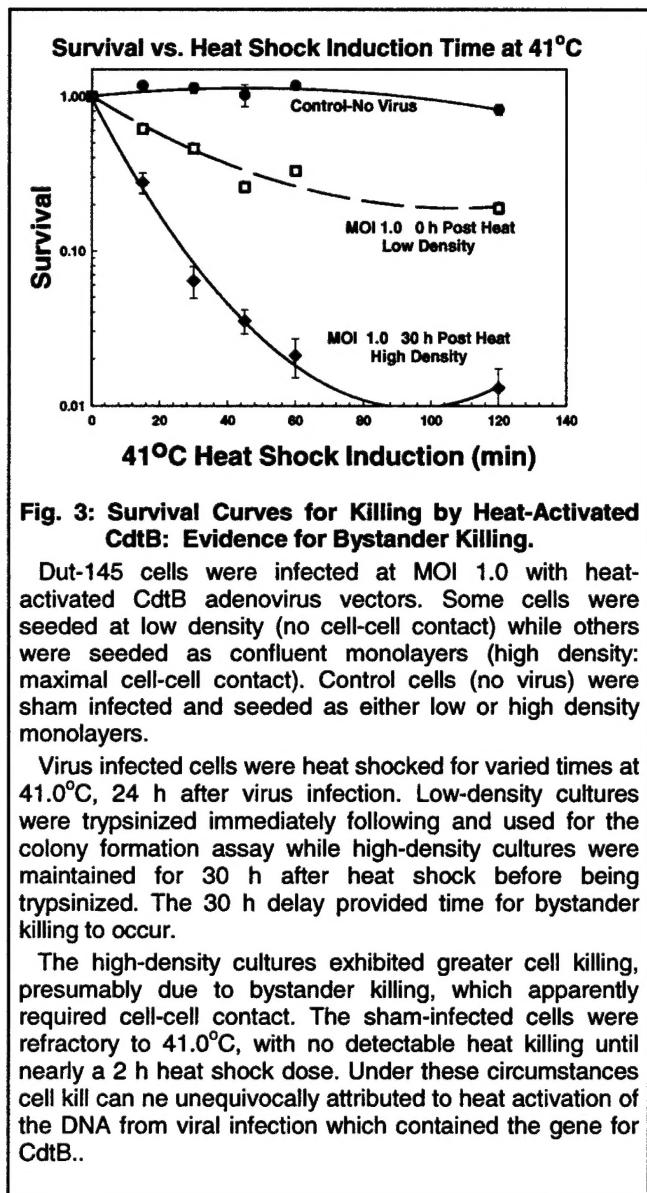
During this reporting period we have succeeded in making complete viral vectors, in usable quantities, using the B component (CdtB) of the Cytolethal Distending Toxin (Cdt) from *E. Coli* under the control of a truncated variant of the heat shock promoter without, the need for incorporation of the radiation induced promoter. Ctd, like the Shigella and Diphtheria toxins, is a tripartite proteotoxin bacterial proteotoxin with both cytotoxic and cell targeting components. In this case the DNA for the A and C components has been removed leaving only the cytotoxic component for incorporation into the viral vector. This

molecule, which shares a high degree of homology with DNase-I, and when produced in the cell is extremely toxic. It kills the cell by the creation of double strand breaks, which also confers radiomimetic properties. Since this vector is intended for the treatment of tumors it is of some importance that it will function in a hypoxic environment, which was confirmed by the data of Figure 2 which demonstrating cell killing and apoptosis in an anaerobic chamber.

**TABLE 1**

% of Cells that Included Trypan Blue 120 h after Mixing CdtB-Expressing Cells with Cells Expressing EGFP

% CdtB Cells	% Trypan Blue Including
50	100
25	100
10	100
5	62
1	11
0	2



**Fig. 3: Survival Curves for Killing by Heat-Activated CdtB: Evidence for Bystander Killing.**

Dut-145 cells were infected at MOI 1.0 with heat-activated CdtB adenovirus vectors. Some cells were seeded at low density (no cell-cell contact) while others were seeded as confluent monolayers (high density: maximal cell-cell contact). Control cells (no virus) were sham infected and seeded as either low or high density monolayers.

Virus infected cells were heat shocked for varied times at 41.0°C, 24 h after virus infection. Low-density cultures were trypsinized immediately following and used for the colony formation assay while high-density cultures were maintained for 30 h after heat shock before being trypsinized. The 30 h delay provided time for bystander killing to occur.

The high-density cultures exhibited greater cell killing, presumably due to bystander killing, which apparently required cell-cell contact. The sham-infected cells were refractory to 41.0°C, with no detectable heat killing until nearly a 2 h heat shock dose. Under these circumstances cell kill can be unequivocally attributed to heat activation of the DNA from viral infection which contained the gene for CdtB..

junctions. However, gap junctions do not normally permit passage of proteins greater than 3-4 kilodaltons and the CdtB protein is approximately 28 kilodaltons. In some ways the observed bystander effect resembles the putative bystander effect from ionizing radiation whose nature is highly controversial at the present time. A great deal of further investigation will be required to understand it but such an effort is extremely worthwhile since bystander activity is critical to effective gene therapy for malignancies.

#### **Detection of the CdtB Protein**

All attempts by or collaborator, Dr. Lawrence Dreyfus and us to detect cellular CdtB expression using Western blots failed, even though the antibody provided by Dr. Dreyfus did detect recombinant CdtB in Western blots. Hence, it was postulated that cells infected with the CdtB adenoviruses simply expressed CdtB levels that were too low to be detected in Western blots. Consequently, we sought other methods to confirm that CdtB was being expressed in mammalian cells that were infected with the CdtB adenovirus and then heat shocked to induce CdtB expression from the HSP70B promoter. To conclusively prove that we were indeed producing the expected gene product it was necessary to resort to other techniques, RT-PCR and immunochemistry, as outlined below.

An unexpected, but extremely important finding, was that this toxin demonstrates a strong bystander effect when cell-cell contact is maintained between infected and uninfected cells, as is demonstrated by in Table 1 above. In this series of experiments infected cells were mixed with uninfected cells in the indicated percentages, held in close contact for 120 hours and then assayed for Trypan Blue dye inclusion. Our conclusion from this data was that it is only necessary to infect 10% of the cells in order to kill the entire population. To pursue this effect further a series of experiments were carried out where all cells were infected at an MOI of 1.0. Under these circumstances statistical considerations dictate that only approximately 63% of the cells are infected with one or more viral particles, the remainder have none. The results of these experiments are shown in Figure 3. When the infected cells were plated sparsely (no cell-cell contact) approximately 80 percent of the cells were killed at the maximum heat dose of 41°C for 2 hours. However when the cells were plated at confluence (high cell-cell contact) 99% of the cells died as assessed by clonogenic assays. The greater cell killing observed for the high-density cell cultures (Fig. 3) supports the existence of CdtB bystander killing and suggests that cell-cell contact is required. We are presently continuing investigations into the nature of this bystander effect. The requirement for cell-cell contact is highly suggestive of the passage of a substance through gap junctions. However, gap junctions do not normally permit passage of proteins greater than 3-4 kilodaltons and the CdtB protein is approximately 28 kilodaltons. In some ways the observed bystander effect resembles the putative bystander effect from ionizing radiation whose nature is highly controversial at the present time. A great deal of further investigation will be required to understand it but such an effort is extremely worthwhile since bystander activity is critical to effective gene therapy for malignancies.

## RT-PCR Detection of CdtB Expression

Dut-145 cells were infected at MOI 5 with the CdtB adenovirus. Half of these cells were heat shocked 24 h later for 1 h at 41.0°C while the remainder was not. RNA was isolated from these cells 9 h later and was subjected to RT-PCR using primers for the CdtB transgene. Non-infected CdtB cells (heated and not) were treated identically to serve as controls.

Figure 4 shows that the RT-PCR assay produced the CdtB DNA fragment only with RNA isolated from cells that were infected with the CdtB virus and then heat shocked. The samples loaded into lanes 7 and 8 were identical to those in, respectively, lanes 5 and 6 except that the reverse transcriptase in the reaction cocktail was inactivated before adding the RNA. This was done to ensure that the DNase treatment destroyed all viral DNA and that the positive signal in lane 6 was due to the presence of CdtB mRNA.

## Immunohistochemical Detection of CdtB

Dr. Dreyfus stated that the antibody that he provided could detect CdtB expressed in cells by using *in situ* immunohistochemical staining. We extended this result to develop a flow cytometric assay that detects CdtB expressed in cells infected with the CdtB adenovirus at MOIs as low as 1 and then heat shocked to induce expression (Fig. 5). These data demonstrate that the CdtB protein can be detected in those cells that were infected with the CdtB adenovirus and then heat shocked to induce expression. The results re-confirm the ability of the HSP70B promoter to control CdtB expression.

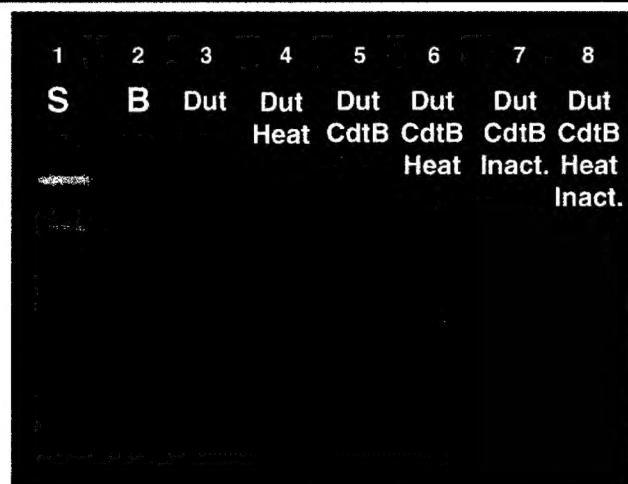


Fig. 4: RT-PCR of CdtB RNA from Dut-145 Cells

Total RNA was isolated from control (3) and heat shocked (4) Dut-145 cells, and from cells infected at MOI 5 with the CdtB adenovirus and then either maintained at 37.0°C (5) or heat shocked at 41.0°C for 1 h to induce CdtB expression before being returned to 37.0°C. RNA samples were subjected to RT-PCR using primers for the CdtB transgene, and the reaction products were run on a 1% agarose gel. Phi phage DNA was run as a standard (S) and the second lane was left blank (B). The samples in lanes 7 and 8 were identical to those in, respectively, lanes 5 and 6, except that the reverse transcriptase was inactivated before adding the RNA and running the reaction.

Only the RNA sample from cells infected with CdtB and then heat shocked to induce expression produced a positive result for CdtB RNA. These data demonstrate heat-induced expression of CdtB in cells infected with the CdtB adenovirus.

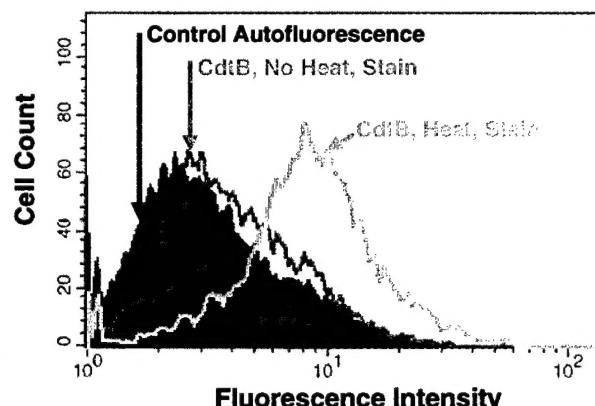


Fig. 5 Immunohistochemical Detection of CdtB.

Cells were fixed with 5% formaldehyde, permeabilized with 0.2% Triton X-110, stained with a polyclonal antibody to CdtB (1:500 dilution), and then counterstained with a secondary antibody labeled with Alexa Flour 488. Cells were then analyzed with a FacScan flow cytometer.

Control cells exhibited detectable auto fluorescence, and the two antibodies caused a mild shift in the fluorescence of infected but unheated cells, which was likely due to nonspecific binding. A clear shift in fluorescence was noted in all cells infected with the CdtB virus and then heat shocked to induce expression. All cells were assayed 48 h after the viral infection and 24 h following the inducing heat shock (41.0°C for 1 h).

## **Key research Accomplishments and Conclusions: Year Two**

- Modifications to the software controlling clinical therapy were completed during this period and are described in detail in the body of the report. These modifications were subjected to the same rigorous testing as the original program and found to be operating within nominal parameters. These modifications simplified the system, making operation of the system much more 'user friendly' for the therapists. It also reduced the maximum number of computers required from three to two.
- Follow-up of the prostate cancer patient treated during year one of the research work continues with no evidence of recurrence.
- An adenoviral vector containing the DNA for a potent proteotoxins (Cytolethal Distending Toxin B (CdtB)) was constructed and produced in sufficient quantities to carry out both *in vitro* and *in vivo* experimentation. This was a major hurdle to overcome and was done without the necessity of including the double heat/radiation promoter switch described in the year one progress report.
- The CdtB containing vector was tested *in vitro* and found to be lethal with a single viral particle infecting a cell. Furthermore, it was discovered that this agent exhibits a remarkable, as yet unexplained, bystander effect which magnifies its potency by a factor of 10 in terms of cell survival. This effect requires cell-cell contact. Such bystander effects are a key requirement for cancer therapy of existing malignancies.
- Experiments were carried out that demonstrate conclusively that the CdtB toxin exhibits DNase-1 like activity and kill by the creation of non-repaired double strand breaks. pH2AX and CHEF gel studies show that the pH2AX foci correspond with DNA double strand break formation and that these phenomena also occur under hypoxic conditions, another key requirement for human tumor therapy.
- The bystander effect requirement for cell-cell contact has led us to develop assays for gap junction activity using Calcein that can be applied in this case. Preliminary results suggest that the bystander activity is transmitted by these gap junctions since pH2AX foci appear in uninfected cells. The full meaning of these observations awaits further experimentation.

## **Reportable Outcomes: Year Two**

One paper has bee accepted for publication in the International Journal of Hyperthermia (see attachment), which directly relates to the subject of this research. The principal author on this was Dr. Elwood Armour, one of the c-investigators on the project.

Armour, E.P. and Raaphorst, P.: Long Duration Mild Hyperthermia and Brachytherapy. Int. J. Hyperthermia, *in press*.

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# Beaumont®

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13 August 2003

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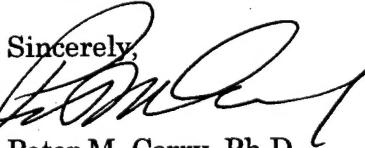
**RE: DAMD 17-01-1-0117, PROJECT TITLE: THERMOBRACHYTHERAPY FOR RECURRENT PROSTATE CANCER**

Dear Ms. Pascal:

Please be aware that the consent form and protocol for the above referenced project have been revised (Revision date: July 21, 2003) to include permanent seed implant eligibility.

Changes to the consent form and protocol are highlighted and described in detail in the attached IRB Amendment Request Form. Copies of the IRB approval letter, revised consent form (Revision date: July 21, 2003 with date stamped IRB approval), and revised protocol (Revision date: July 21, 2003) are enclosed. *NOTE: As of April 1, 2003, our IRB began using an approval date stamp on all consent forms.*

Should you have any questions or require additional information, please feel free to contact me at 248/551-2560 or via e-mail at [pcorry@beaumont.edu](mailto:pcorry@beaumont.edu).

Sincerely,  
  
Peter M. Corry, Ph.D.

:mlf

Enclosures: IRB Amendment Request Form  
IRB Approval Letter (Dated August 4, 2003)  
HIC 98-54 Consent Form (Revised July 21, 2003 w/ date stamped IRB approval)  
HIC 98-54 Protocol (Revised July 21, 2003)

## APPENDIX - PAGE 11



William Beaumont Hospital  
Royal Oak

Human Investigation Committee  
Richard L. Lucarotti, Pharm.D.  
Chairman

August 4, 2003

Alvaro Martinez, MD  
Department of Radiation Oncology  
William Beaumont Hospital  
Royal Oak, Michigan 48073

Dear Investigator:

**RE: Project: HIC-1998-054 (Amendment received: July 25, 2003)**

**PILOT STUDY TESTING THE TECHNICAL FEASIBILITY AND TOXICITY OF HIGH  
DOSE RATE BRACHYTHERAPY COMBINED WITH HYPERTERMIA TO TREAT  
PROSTATE CANCER RECURRENCES AFTER EXTERNAL BEAM IRRADIATION OR  
PERMANENT SEED IMPLANT FAILURE**

I have reviewed the amendment (dated July 22, 2003) revising the exclusion criteria to the protocol and modifying the title of the consent form (dated July 21, 2003) as suggested by the principal investigator. I believe the amendment involves no more than minimal risk to human subjects as detailed in Docket #87N-0032 of the Federal Register (6/18/91).

The amendment request has been extended **FULL APPROVAL** under the Expedited Review policy (21 CFR 56.110) of the Human Investigation Committee.

All amendments to the protocol, except those necessary to eliminate apparent immediate hazards to human subjects, may not be initiated without review and approval by the Human Investigation Committee. Note: any deviation from protocol must be reported immediately.

Sincerely,

Richard L. Lucarotti, Pharm. D.  
Chairman  
Human Investigation Committee  
/js-amendapp

**APPENDIX -  
PAGE 12**

# International Journal of HYPERTHERMIA

RECEIVED JUL 08

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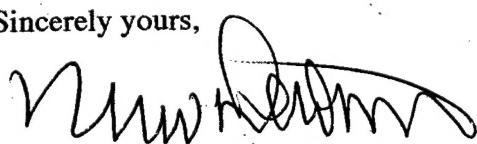
RE: International Journal of Hyperthermia manuscript #03-17

*"Long duration mild temperature hyperthermia and brachytherapy"*

Dear Dr. Armour:

I am pleased to inform you that the above referenced manuscript has now been accepted for publication in the International Journal of Hyperthermia. The paper has been forwarded to the publisher. You should expect to see galley proofs within 3 months. If you have not received them by that time, please contact my office and we will track the manuscript for you.

Sincerely yours,



Mark W. Dewhirst, D.V.M., Ph.D.

APPENDIX -  
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